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(54) Title: A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT

(57) Abstract

A variant of human Kunitz-type protease inhibitor domain I of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence X^1 Cys Ala Phe Lys Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe X^{10} Tyr Gly Gly Cys X^{11} X^{12} X^{13} Gln Asn Arg Phe X^{14} Ser Leu Glu Glu Cys X^{15} X^{16} Met Cys Thr Arg X^{17} (SEQ ID No. 1), wherein X^1 represents H or 1-7 naturally occurring amino acid residues except Cys, X^2-X^{16} each independently represents a naturally occurring amino acid residue, and X^{17} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{17} is different from the corresponding amino acid residue of the native sequence.

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A HUMAN KUNITZ-TYPE PROTEASE INHIBITOR VARIANT

FIELD OF INVENTION

The present invention relates to a variant of a human Kunitz-type protease inhibitor domain, DNA encoding the variant, a method of producing the variant and a pharmaceutical composition containing the variant.

BACKGROUND OF THE INVENTION

Polymorphonuclear leukocytes (neutrophils or PMNs) and mononuclear phagocytes (monocytes) play an important part in tissue injury, infection, acute and chronic inflammation and wound healing. The cells migrate from the blood to the site of inflammation and, following appropriate stimulation, they release oxidant compounds ($O_2\bullet$, O_2-, H_2O_2 and HOCl) as well as granules containing a variety of proteolytic enzymes. The secretory granules contain, i.a., alkaline phosphatase, metalloproteinases such as gelatinase and collagenase and serine proteases such as neutrophil elastase, cathepsin G and proteinase 3.

Latent metalloproteinases are released together with tissue inhibitor of metalloproteinase (TIMP). The activation mechanism has not been fully elucidated, but it is likely that oxidation of thiol groups and/or proteolysis play a part in the process. Also, free metalloproteinase activity is dependent on inactivation of TIMP.

In the azurophil granules of the leukocytes, the serine proteases neutrophil elastase, cathepsin G and proteinase-3 are packed as active enzymes complexed with glucosaminoglycans. These complexes are inactive but dissociate on secretion to release the active enzymes. To neutralise the protease activity, large amounts of the inhibitors α_1-proteinase inhibitor (α_1-PI)

and α_1-chymotrypsin inhibitor (α_1-ChI) are found in plasma. However, the PMNs are able to inactivate the inhibitors locally. Thus, α_1-PI which is the most important inhibitor of neutrophil elastase is sensitive to oxidation at the reactive centre (Met-358) by oxygen metabolites produced by triggered PMNs. This reduces the affinity of α_1-PI for neutrophil elastase by approximately 2000 times.

After local neutralisation of α_1-PI, the elastase is able to degrade a number of inhibitors of other proteolytic enzymes. Elastase cleaves α_1-ChI and thereby promotes cathepsin G activity. It also cleaves TIMP, resulting in tissue degradation by metalloproteinases. Furthermore, elastase cleaves antithrombin III and heparin cofactor II, and tissue factor pathway inhibitor (TFPI) which probably promotes clot formation. On the other hand, the ability of neutrophil elastase to degrade coagulation factors is assumed to have the opposite effect so that the total effect of elastase is unclear. The effect of neutrophil elastase on fibrinolysis is less ambiguous. Fibrinolytic activity increases when the elastase cleaves the plasminogen activator inhibitor and the α_2 plasmin inhibitor. Besides, both of these inhibitors are oxidated and inactivated by O_2 metabolites.

PMNs contain large quantities of serine proteases, and about 200 mg of each of the leukocyte proteases are released daily to deal with invasive agents in the body. Acute inflammation leads to a many-fold increase in the amount of enzyme released. Under normal conditions, proteolysis is kept at an acceptably low level by large amounts of the inhibitors α_1-PI, α_1-ChI and α_2 macroglobulin. There is some indication, however, that a number of chronic diseases is caused by pathological proteolysis due to overstimulation of the PMNs, for instance caused by autoimmune response, chronic infection, tobacco smoke or other irritants, etc.

Aprotinin (bovine pancreatic trypsin inhibitor) is known to

operations). Besides, aprotinin is a bovine protein which may therefore contain one or more epitopes which may give rise to an undesirable immune response on administration of aprotinin to humans.

It is therefore an object of the present invention to identify human protease inhibitors of the same type as aprotinin (i.e. Kunitz-type inhibitors) with a similar inhibitor profile or modified to exhibit a desired inhibitor profile.

SUMMARY OF THE INVENTION

The present invention relates to a variant of human Kunitz-type protease inhibitor domain I of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence

X^1 Cys Ala Phe Lys Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe X^{10} Tyr Gly Gly Cys X^{11} X^{12} X^{13} Gln Asn Arg Phe X^{14} Ser Leu Glu Glu Cys X^{15} X^{16} Met Cys Thr Arg X^{17} (SEQ ID No. 1)

wherein X^1 represents H or 1-7 naturally occurring amino acid residues except Cys, X^2-X^{16} each independently represents a naturally occurring amino acid residue except Cys, and X^{17} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{17} is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e. Ala, Val, Leu, Ile Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

TFPI, also known as extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI), has been

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TFPI, also known as extrinsic pathway inhibitor (EPI) or lipoprotein associated coagulation inhibitor (LACI), has been

isolated by Broze et al. (Proc. Natl. Acad. Sci. USA 84, 1987, pp. 1886-1890 and EP 300 988) and the gene coding for the protein has been cloned, cf. EP 318 451. Analysis of the secondary structure of the protein has shown that the protein has three Kunitz-type inhibitor domains, from amino acid 22 to amino acid 79 (I), from amino acid 93 to amino acid 150 (II) and from amino acid 185 to amino acid 242 (III). Kunitz-type domain I of TFPI has been shown to bind TF/FVIIa, while Kunitz-type domain II has been shown to bind to FXa (Girard et al., Nature 338, 1989, pp. 518-520).

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor profile of TFPI Kunitz-type domain I so that it preferentially inhibits neutrophil elastase, cathepsin G and/or proteinase-3. Furthermore, it may be possible to construct variants which specifically inhibit enzymes involved in coagulation or fibrinolysis (e.g. plasmin or plasma kallikrein) or the complement cascade.

One advantage of TFPI Kunitz-type domain I is that it has a negative net charge as opposed to aprotinin which, as indicated above, has a strongly positive net charge. It is therefore possible to construct variants of the invention with a lower positive net charge than aprotinin, thereby reducing the risk of kidney damage on administration of large doses of the variants. Another advantage is that, contrary to aprotinin, it is a human protein (fragment) so that undesired immunological reactions on administration to humans are significantly reduced.

DETAILED DISCLOSURE OF THE INVENTION

Examples of preferred variants of Kunitz-type domain I of TFPI are variants wherein X^1 is Ser-Phe or Met-His-Ser-Phe; or wherein X^2 is an amino acid residue selected from the group consisting of Ala, Arg, Thr, Asp, Pro, Glu, Lys, Gln, Ser, Ile and Val, in particular wherein X^2 is Thr or Asp; or wherein X^3 is an amino

acid residue selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile, in particular wherein X^3 is Pro or Ile; or wherein X^4 is an amino acid residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala, in particular wherein X^4 is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg; or wherein X^5 is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp, in particular wherein X^5 is Ala, Thr, Asp or Gly; or wherein X^6 is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met, in particular wherein X^6 is Arg, Phe, Ala, Ile, Leu or Tyr; or wherein X^7 is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe, in particular wherein X^7 is Ile; or wherein X^8 is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe, in particular wherein X^8 is Ile or Lys; or wherein X^9 is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu, in particular wherein X^9 is Arg; or wherein X^{10} is an amino acid residue selected from the group consisting of Gln, Pro, Phe, Ile Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val, in particular wherein X^{10} is Val or Ile; or wherein X^{11} is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn, in particular wherein X^{11} is Arg or Glu; or wherein X^{12} is Ala or Gly; or wherein X^{13} is an amino acid residue selected from the group consisting of Lys, Asn and Asp, in particular wherein X^{13} is Lys or Asn; or wherein X^{14} is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys, in particular wherein X^{14} is Lys or Glu; or wherein X^{15} is Lys, Met, Glu or Leu; or wherein X^{16} is Lys, Ala, Asn or Glu; or wherein X^{17} is Asp. In a preferred embodiment, X^1 is Met-His-Ser-Phe and X^{17} is Asp, while X^2-X^{16} are as defined above.

Variants of TFPI Kunitz-type domain I of the invention should preferably not contain a Met residue in the protease binding

region (i.e. the amino acid residues represented by X^3-X^{14}). By analogy to α1-PI described above, a Met residue in any one of these positions would make the inhibitor sensitive to oxidative inactivation by oxygen metabolites produced by PMNs, and conversely, lack of a Met residue in these positions should render the inhibitor more stable in the presence of such oxygen metabolites.

A currently preferred variant of the invention is one in which one or more of the amino acid residues located at the protease-binding site of the Kunitz domain (i.e. one or more of X^3-X^{14} corresponding to positions 13, 15, 16, 17, 18, 19, 20, 34, 39, 40, 41 and 46 of aprotinin) are substituted to the amino acids present in the same positions of native aprotinin. This variant comprises the following amino acid sequence

Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala Arg Ile Ile Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe Val Tyr Gly Gly Cys Arg Ala Lys Gln Asn Arg Phe Lys Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp (SEQ ID No. 2).

In another aspect, the invention relates to a DNA construct encoding a human Kunitz-type inhibitor domain variant according to the invention. The DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Alternatively, it is possible to use genomic or cDNA coding for TFPI Kunitz-type domain I (e.g. obtained by screening a genomic or cDNA library for DNA coding for TFPI using synthetic oligonucleotide probes and isolating the DNA sequence coding for

domain I therefrom). The DNA sequence is modified at one or more sites corresponding to the site(s) at which it is desired to introduce amino acid substitutions, e.g. by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures.

In a still further aspect, the invention relates to a recombinant expression vector which comprises a DNA construct of the invention. The recombinant expression vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the TFPI Kunitz-type domain I variant of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the TFPI Kunitz-type domain I variant of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic

Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the *tpi*A promoter.

The DNA sequence encoding the TFPI Kunitz-type domain I variant of the invention may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication, or (when the host cell is a yeast cell) the yeast plasmid 2μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate, or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130.

The procedures used to ligate the DNA sequences coding for the TFPI Kunitz-type domain I variant of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory

Manual, Cold Spring Harbor, New York, 1989).

The host cell into which the expression vector of the invention is introduced may be any cell which is capable of producing the TFPI Kunitz-type domain I variant of the invention and is preferably a eukaryotic cell, such as a mammalian, yeast or fungal cell.

The yeast organism used as the host cell according to the invention may be any yeast organism which, on cultivation, produces large quantities of the TFPI Kunitz-type domain I variant of the invention. Examples of suitable yeast organisms are strains of the yeast species Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe or Saccharomyces uvarum. The transformation of yeast cells may for instance be effected by protoplast formation followed by transformation in a manner known per se.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells may be used as host cells of the invention. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 238 023.

The present invention further relates to a method of producing a TFPI Kunitz-type domain I variant according to the invention, the method comprising culturing a cell as described above under conditions conducive to the expression of the variant and recovering the resulting variant from the culture.

The medium used to cultivate the cells may be any conventional medium suitable for growing mammalian cells or fungal (including yeast) cells, depending on the choice of host cell. The variant will be secreted by the host cells to the growth medium and may be recovered therefrom by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

The present invention also relates to a pharmaceutical composition comprising a TFPI Kunitz-type domain I variant of the invention together with a pharmaceutically acceptable carrier or excipient. In the composition of the invention, the variant may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution.

It has surprisingly been found that the TFPI Kunitz-type domain I is in itself capable of inhibiting Cathepsin G. The invention therefore also relates to a pharmaceutical composition for the inhibition of Cathepsin G, the composition comprising human Kunitz-type protease inhibitor domain I of TFPI or a variant thereof as described above and a pharmaceutically acceptable carrier or excipient.

The TFPI Kunitz-type domain I variant of the invention is therefore contemplated to be advantageous to use for the therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitor profiles, in particular those which necessitate the use of large aprotinin doses. Therapeutic applications for which the use of the variant of the invention is indicated as a result of its ability to inhibit human serine proteases, e.g. trypsin, plasmin, kallikrein, elastase, cathepsin G and proteinase-3, include (but are not limited to) acute pancreatitis, inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, shock (including shock lung) and conditions involving hyperfibrinolytic haemorrhage, emphysema, rheumatoid arthritis, adult respiratory distress syndrome, chronic inflammatory bowel disease and psoriasis, in other words diseases presumed to be caused by pathological proteolysis by elastase, cathepsin G and proteinase-3 released from triggered PMNs.

Furthermore, the present invention relates to the use of TFPI Kunitz-type inhibitor domain I or a variant thereof as described above for the preparation of a medicament for the prevention or therapy of diseases or conditions associated with pathological proteolysis by proteases released from overstimulated PMNs. As indicated above, it may be an advantage of administer heparin concurrently with the TFPI Kunitz-type inhibitor domain I or variant.

Apart from the pharmaceutical use indicated above, TFPI Kunitz-type domain II or a variant thereof as specified above may be used to isolate useful natural substances, e.g. proteases or receptors from human material, which bind directly or indirectly to TFPI Kunitz-type domain II, for instance by screening assays or by affinity chromatography.

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLES

General Methods

Standard DNA techniques were carried out as described (Sambrook, J., Fritch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.). Synthetic oligonucleotides were prepared on an automatic DNA synthesizer (380B, Applied Biosystems) using phosphoramidite chemistry on a controlled pore glass support (Beaucage, S.L., and Caruthers, M.H., Tetrahedron Letters 22, (1981) 1859-1869). DNA sequence determinations were performed by the dideoxy chain-termination technique (Sanger, F., Micklen, S., and Coulson, A.R., Proc.Natl. Acad.Sci. USA 74 (1977) 5463-5467). Polymerase chain reactions (PCR) were performed on a DNA Thermal Cycler (Perkin Elmer Cetus).

Amino acid analysis was carried out after hydrolysis in 6M HCl at 110°C in vacuum-sealed tubes for 24 hours. Analysis was performed on a Beckman 121MB automatic amino acid analyzer modified for microbore operation.

N-terminal amino acid sequence analysis was obtained by automated Edman degradation using an Applied Biosystems 470A gas-phase sequencer. Analysis by on-line reverse phase HPLC was performed for the detection and quantitation of the liberated pTH amino acids from each sequencer cycle.

Molecular weight determination was obtained on a BIO-ION 20 plasma desorption mass spectrometer (PDMS) equipped with a flight tube of approximately 15 cm and operated in positive mode. Aliquots of 5 µl were analyzed at an accelerating voltage set to 15 kV and ions were collected for 5 million fission events. The accuracy on assigned molecular ions is approximately 0.1% for well defined peaks, otherwise somewhat less.

Example 1

Production of the first Kunitz domain of tissue factor pathway inhibitor, TFPI-1, from yeast strain KFN-1651

cDNA encoding full length TFPI was isolated from the human liver derived cell line HepG2 (ATCC HB 8065) and inserted as a 0.9 kb BamHI-XbaI fragment into a mammalian expression vector, pKFN-1168, as described (Pedersen, A.H., Nordfang, O., Norris, F., Wiberg, F.C., Christensen, P.M., Moeller, K.B., Meidahl-Pedersen, J., Beck, T.C., Norris, K., Hedner, U., and Kisiel, W. 1990, J.Biol.Chem. 265, 16786-16793). The DNA sequence of the insert is given in SEQ ID No. 3. TFPI-1 is encoded by nucleotides 152-325 as indicated.

TFPI-1: 0.1 μg of the 0.9 kb BamHI-XbaI fragment from pKFN-1168 was used as a template in a PCR reaction containing 100 pmole each of the primers NOR-2524 (GCTGAGAGATTGGAGAAGAGAATGCATTCATTTTGTGC) and NOR-2525 (TAATCCTTCTAGATTAATCTCTTGTACACAT). The 17 3'-terminal bases of NOR-2524 are identical to bases 152 to 168 in the TFPI-1 gene in SEQ ID No. 3, and the 21 5'-terminal bases are identical to bases 215 to 235 in the synthetic leader gene (see SEQ ID No. 5) from pKFN-1000 described below. Primer NOR-2525 is complementary to bases 311 to 325 in SEQ ID No. 3 and has a 5' extension containing a translation stop codon followed by an XbaI site.

The PCR reaction was performed in a 100 μl volume using a commercial kit (GeneAmp, Perkin Elmer Cetus) and the following cycle: 94° for 20 sec, 50° for 20 sec, and 72° for 30 sec. After 19 cycles a final cycle was performed in which the 72° step was maintained for 10 min. The PCR product, a 211 bp fragment, was isolated by electrophoresis on a 2% agarose gel.

Signal-leader: 0.1 μg of a 0.7 kb PvuII fragment from pKFN-1000 described below was used as a template in a PCR reaction containing 100 pmole each of the primers NOR-1478 (GTAAACGACGGCCAGT) and NOR-2523 (TCTCTTCTCCAATCTCTCAGC). NOR-

1478 is matching a sequence just upstream of the EcoRI site in SEQ ID No. 5. Primer NOR-2523 is complementary to the 17 3'-terminal bases of the synthetic leader gene of pKFN-1000, see SEQ ID No. 5. The PCR reaction was performed as described above, resulting in a 257 bp fragment.

Plasmid pKFN-1000 is a derivative of plasmid pTZ19R (Mead, D.A., Szczesna-Skorupa, E. and Kemper, B., Prot. Engin. 1 (1986) 67-74) containing DNA encoding a synthetic yeast signal-leader peptide.

Plasmid pKFN-1000 is described in WO 90/10075. The DNA sequence of 235 bp downstream from the EcoRI site of pKFN-1000 and the encoded amino acid sequence of the synthetic yeast signal-leader is given in SEQ ID No. 5.

Signal-leader-TFPI-1: Approx. 0.1 µg of each of the two PCR-fragments described above were mixed. A PCR reaction was performed using 100 pmole each of primers NOR-1478 and NOR-2525 and the following cycle: 94° for 1 min, 50° for 2 min, and 72° for 3 min. After 16 cycles a final cycle was performed in which the 72° step was maintained for 10 min.

The resulting 443 bp fragment was purified by electrophoresis on a 1% agarose gel and then digested with EcoRI and XbaI. The resulting 412 bp fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636. Plasmid pMT636 is described in WO 89/01968.

pMT636 is an *E. coli* - *S. cerevisiae* shuttle vector containing the *Schizosaccharomyces pombe* TPI gene (POT) (Russell, P.R., Gene 40 (1985) 125-130), the *S. cerevisiae* triosephosphate isomerase promoter and terminator, TPI_P and TPI_T (Alber, T., and Kawasaki, G., J.Mol.Appl.Gen. 1 (1982), 419-434).

The ligation mixture was used to transform a competent *E. coli* strain (r^-, m^+) selecting for ampicillin resistance. DNA

sequencing showed that plasmids from the resulting colonies contained the correct DNA sequence for TFPI-1 correctly fused to the synthetic yeast signal-leader gene.

One plasmid, pKFN-1603, was selected for further use. The construction of plasmid pKFN-1603 is illustrated in fig. 1.

The expression cassette of plasmid pKFN-1603 contains the following sequence:

TPI_P - KFN1000 signal-leader - TFPI1 - TPI_T

The DNA sequence of the 412 bp EcoRI-XbaI fragment from pKFN-1603 is shown in SEQ ID No. 7.

Yeast transformation: S. cerevisiae strain MT663 (E2-7B XE11-36 a/α, Δtpi/Δtpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an O.D. at 600 nm of 0.6.

100 ml of culture was harvested by centrifugation, washed with 10 ml of water, recentrifugated and resuspended in 10 ml of a solution containing 1.2 M sorbitol, 25 mM Na_2EDTA pH = 8.0 and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate, pH 0 5.8, and 2 mg Novozym®234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris HCl (Tris = Tris(hydroxymethyl)aminomethane) pH = 7.5) and resuspended in 2 ml of CAS. For transformation, 0.1 μg of plasmid pKFN-1603 and left at room temperature for 15 minutes. 1 ml of (20% polyethylene glycol 4000, 20 mM $CaCl_2$, 10 mM $CaCl_2$, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for a further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 μg/ml leucine) and incubated at 30°C for

2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory (1982)) containing 1.2 M sorbitol plus 2.5%agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium.

Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant KFN-1651 was selected for further characterization.

Fermentation: Yeast strain KFN-1651 was grown on YPD medium (1% yeast extract, 2% peptone (from Difco Laboratories), and 3% glucose). A 1 liter culture of the strain was shaken at 30°C to an optical density at 650 nm of 24. After centrifugation the supernatant was isolated.

The yeast supernatant was adjusted to pH 3.0 with 5% acetic acid and phosphoric acid and applied a column of S-Sepharose Fast Flow (Pharmacia) and equilibrated with 50 mM formic acid, pH 3.7. After wash with equilibration buffer, the HKI-domain was eluted with 1 M sodium chloride. Desalting was obtained on a Sephadex G-25 column (Pharmacia) equilibrated and eluted with 0.1% ammonium hydrogen carbonate, pH 7.9. After concentraton by vacuum centifugation and adjustment of pH 3.0 further purification was performed on a Mono S column (Pharmacia) equilibrated with 50 mM formic acid, pH 3.7. After wash with equilibration buffer, gradient elution was carried out from 0 - 1 M sodium chloride in equilibration buffer. Final purification was performed by reverse phase HPLC on a Vydac C4 column (The Separation Group, CA) with gradient elution from 5-55% acetonitrile, 0.1% TFA. The purified product was lyophilised by vacuum centrifugation and redissolved in water.

Aliquots were analysed by mass PD-mass spectrometry (found: MW 6853,5, calculated: MW 6853-8) and N-terminal amino acid

sequencing for 45 Edman degradation cycles confirmed the primary structure of the TFPI-1 domain (Table 1)

Table 1

N-Terminal Sequence Analysis of TFPI-1

Approx. 350 pmol of KFN1651 (HPLC-fraction 18#920327) was analysed.

The repetitive yield was xx.x %. Sequencer run#1575.

Cycle No.	Amino acid	Yield (pmol)	Cycle No.	Amino acid	Yield (pmol)
1	Met	250	31	Glu	19
2	His	47	32	Glu	25
3	Ser	69	33	Phe	27
4	Phe	301	34	Ile	18
5	Cys	-	35	Tyr	16
6	Ala	226	36	Gly	26
7	Phe	201	37	Gly	36
8	Lys	201	38	Cys	-
9	Ala	216	39	Glu	11
10	Asp	105	40	Gly	25
11	Asp	117	41	Asn	14
12	Gly	148	42	Gln	15
13	Pro	62	43	Asn	19
14	Cys	-	44	Arg	15
15	Lys	78	45	Phe	12
16	Ala	98	46	Glu	
17	Ile	75	47	Ser	
18	Met	57	48	Leu	
19	Lys	69	49	Glu	
20	Arg	48	50	Glu	
21	Phe	63	51	Cys	
22	Phe	90	52	Lys	
23	Phe	99	53	Lys	
24	Asn	46	54	Met	
25	Ile	50	55	Cys	
26	Phe	56	56	Thr	
27	Thr	25	57	Arg	
28	Arg	35	58	Asp	
29	Gln	33			
30	Cys	-			

The PTH-derivative of Cys is not identified, e.g. cycles 5, 14, 30 and 38.

The sequenater was stopped after 60 cycles and the sequence could be deduced for the first 45 amino acids.

Example

Inhibition of serine proteinases by TFPI (domain I) KFN 1651

KFN 1651 was purified from yeast culture medium. The concentration of KFN 1651 was determined from the absorbance at 214 nm using BPTI as a standard. Porcine trypsin and human recombinant factor VIIa was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark), bovine chymotrypsin (TLCK treated) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human truncated recombinant tissue factor was obtained from Corvas (San Diego, CA, USA).

Human neutrophil cathepsin G was purified from extracts of PMNs according to the method described by Baugh and Travis (Biochemistry 15 (1976) 836-843). Peptidyl nitroanilide substrates, S2251, S2586, S2288 were from Kabi (Stockholm, Sweden). S7388 was from Sigma Chemical Co. (St. Louis, MO, USA) and FXa-1 was from NycoMed (Oslo, Norway).

Serine proteinases were incubated with various concentrations of KFN 1651 for 30 min. Substrate was then added and residual proteinase activity was measured at 405 nm. The results are shown in Table 2.

Unmodified TFPI Kunitz domain I (KFN 1651) was found to be an inhibitor of trypsin, chymotrypsin, meutrophil Cathepsin G and factor VIIa/tissue factor.

Table 2

Protease	Apparent Ki
Trypsin	18×10^{-9} M
Chymotrypsin	1.2×10^{-6} M
Cathepsin G	87×10^{-9} M
Factor VIIa/TF	150×10^{-9} M

50 mM Tris Cl, 100 mM NaCl, pH 7.4.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(I) TELEX: 37304

(ii) TITLE OF INVENTION: A Human Kunitz-Type Protease Inhibitor Variant

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Xaa Cys Ala Phe Lys Ala Asp Xaa Gly Xaa Cys Xaa Xaa Xaa Xaa Xaa
1               5                   10                  15

Xaa Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe Xaa Tyr
            20                  25                  30

Gly Gly Cys Xaa Xaa Xaa Gln Asn Arg Phe Xaa Ser Leu Glu Glu Cys
        35                  40                  45

Xaa Xaa Met Cys Thr Arg Xaa
    50                  55
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala
1               5                   10                  15

Arg Ile Ile Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu
            20                  25                  30

Phe Val Tyr Gly Gly Cys Arg Ala Lys Gln Asn Arg Phe Glu Ser Leu
        35                  40                  45

Glu Glu Cys Lys Lys Met Cys Thr Arg Asp
    50                  55
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 945 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 152..325

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GGATCCGAAT TCCACCATGA AGAAAGTACA TGCACTTTGG GCTTCTGTAT GCCTGCTGCT      60

TAATCTTGCC CCTGCCCCTC TTAATGCTGA TTCTGAGGAA GATGAAGAAC ACACAATTAT     120

CACAGATACG GAGTTGCCAC CACTGAAACT T ATG CAT TCA TTT TGT GCA TTC       172
                                  Met His Ser Phe Cys Ala Phe
                                  1               5

AAG GCG GAT GAT GGC CCA TGT AAA GCA ATC ATG AAA AGA TTT TTC TTC      220
Lys Ala Asp Asp Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe
        10                  15                  20
```

AAT ATT TTC ACT CGA CAG TGC GAA GAA TTT ATA TAT GGG GGA TGT GAA 268
Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu
25 30 35

GGA AAT CAG AAT CGA TTT GAA AGT CTG GAA GAG TGC AAA AAA ATG TGT 316
Gly Asn Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys
40 45 50 55

ACA AGA GAT AATGCAAACA GGATTATAAA GACAACATTG CAACAAGAAA 365
Thr Arg Asp

AGCCAGATTT CTGCTTTTTG GAAGAAGATC CTGGAATATG TCGAGGTTAT ATTACCAGGT 425

ATTTTTATAA CAATCAGACA AAACAGTGTG AACGTTTCAA GTATGGTGGA TGCCTGGGCA 485

ATATGAACAA TTTTGAGACA CTGGAAGAAT GCAAGAACAT TTGTGAAGAT GGTCCGAATG 545

GTTTCCAGGT GGATAATTAT GGAACCCAGC TCAATGCTGT GAATAACTCC CTGACTCCGC 605

AATCAACCAA GGTTCCCAGC CTTTTTGAAT TTCACGGTCC CTCATGGTGT CTCACTCCAG 665

CAGACAGAGG ATTGTGTCGT GCCAATGAGA ACAGATTCTA CTACAATTCA GTCATTGGGA 725

AATGCCGCCC ATTTAAGTAC AGTGGATGTG GGGGAAATGA AAACAATTTT ACTTCCAAAC 785

AAGAATGTCT GAGGGCATGT AAAAAAGGTT TCATCCAAAG AATATCAAAA GGAGGCCTAA 845

TTAAAACCAA AAGAAAAAGA AAGAAGCAGA GAGTGAAAAT AGCATATGAA GAGATCTTTG 905

TTAAAAATAT GTGAATTTGT TATAGCAATG TAACTCTAGA 945

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala
1 5 10 15

Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu
20 25 30

Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser Leu
35 40 45

Glu Glu Cys Lys Lys Met Cys Thr Arg Asp
50 55

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 235 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..235

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT     60

ATAAACGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC        109
                  Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                   1               5                  10

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG      157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
             15                  20                  25

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC      205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
         30                  35                  40

GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA                              235
Val Ala Met Ala Glu Arg Leu Glu Lys Arg
     45                  50
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
  1               5                  10                  15

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
             20                  25                  30
```

```
Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
        35                  40                  45

Arg Leu Glu Lys Arg
    50
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic/human

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 77..409

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 77..235

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 236..409

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
GAATTCCATT CAAGAATAGT TCAAACAAGA AGATTACAAA CTATCAATTT CATACACAAT        60

ATAAACGACC AAAAGA ATG AAG GCT GTT TTC TTG GTT TTG TCC TTG ATC           109
                  Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile
                  -53         -50                     -45

GGA TTC TGC TGG GCC CAA CCA GTC ACT GGC GAT GAA TCA TCT GTT GAG         157
Gly Phe Cys Trp Ala Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu
        -40                 -35                 -30

ATT CCG GAA GAG TCT CTG ATC ATC GCT GAA AAC ACC ACT TTG GCT AAC         205
Ile Pro Glu Glu Ser Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn
    -25                 -20                 -15

GTC GCC ATG GCT GAG AGA TTG GAG AAG AGA ATG CAT TCA TTT TGT GCA         253
Val Ala Met Ala Glu Arg Leu Glu Lys Arg Met His Ser Phe Cys Ala
-10                 -5                  1               5

TTC AAG GCG GAT GAT GGC CCA TGT AAA GCA ATC ATG AAA AGA TTT TTC         301
Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe
                10                  15                  20
```

```
TTC AAT ATT TTC ACT CGA CAG TGC GAA GAA TTT ATA TAT GGG GGA TGT      349
Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys
         25                  30                  35

GAA GGA AAT CAG AAT CGA TTT GAA AGT CTG GAA GAG TGC AAA AAA ATG      397
Glu Gly Asn Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met
     40                  45                  50

TGT ACA AGA GAT TAATCTAGA                                            418
Cys Thr Arg Asp
 55
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Met Lys Ala Val Phe Leu Val Leu Ser Leu Ile Gly Phe Cys Trp Ala
-53         -50                 -45                 -40

Gln Pro Val Thr Gly Asp Glu Ser Ser Val Glu Ile Pro Glu Glu Ser
    -35                 -30                 -25

Leu Ile Ile Ala Glu Asn Thr Thr Leu Ala Asn Val Ala Met Ala Glu
    -20                 -15                 -10

Arg Leu Glu Lys Arg Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
 -5                  1               5                  10

Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Thr
            15                  20                  25

Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
        30                  35                  40

Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp
    45                  50                  55
```

CLAIMS

1. A variant of human Kunitz-type protease inhibitor domain I of tissue factor pathway inhibitor (TFPI), the variant comprising the following amino acid sequence

X^1 Cys Ala Phe Lys Ala Asp X^2 Gly X^3 Cys X^4 X^5 X^6 X^7 X^8 X^9 Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe X^{10} Tyr Gly Gly Cys X^{11} X^{12} X^{13} Gln Asn Arg Phe X^{14} Ser Leu Glu Glu Cys X^{15} X^{16} Met Cys Thr Arg X^{17} (SEQ ID No. 1)

wherein X^1 represents H or 1-7 naturally occurring amino acid residues except Cys, X^2-X^{16} each independently represents a naturally occurring amino acid residue, and X^{17} represents OH or 1-5 naturally occurring amino acid residues except Cys, with the proviso that at least one of the amino acid residues X^1-X^{17} is different from the corresponding amino acid residue of the native sequence.

2. A variant according to claim 1, wherein X^1 is Ser-Phe or Met-His-Ser-Phe.

3. A variant according to claim 1, wherein X^2 is an amino acid residue selected from the group consisting of Ala, Arg, Thr, Asp, Pro, Glu, Lys, Gln, Ser, Ile and Val.

4. A variant according to claim 3, wherein X^2 is Thr or Asp.

5. A variant according to claim 1, wherein X^3 is an amino acid residue selected from the group consisting of Pro, Thr, Leu, Arg, Val and Ile.

6. A variant according to claim 5, wherein X^3 is Pro or Ile.

7. A variant according to claim 1, wherein X^4 is an amino acid

residue selected from the group consisting of Lys, Arg, Val, Thr, Ile, Leu, Phe, Gly, Ser, Met, Trp, Tyr, Gln, Asn and Ala.

8. A variant according to claim 7, wherein X^4 is Lys, Val, Leu, Ile, Thr, Met, Gln or Arg.

9. A variant according to claim 1, wherein X^5 is an amino acid residue selected from the group consisting of Ala, Gly, Thr, Arg, Phe, Gln and Asp.

10. A variant according to claim 9, wherein X^5 is Ala, Thr, Asp or Gly.

/6

11. A variant according to claim 1, wherein X^6 is an amino acid residue selected from the group consisting of Arg, Ala, Lys, Leu, Gly, His, Ser, Asp, Gln, Glu, Val, Thr, Tyr, Phe, Asn, Ile and Met.

12. A variant according to claim 11, wherein X^6 is Arg, Phe, Ala, Ile, Leu or Tyr.

/7

13. A variant according to claim 1, wherein X^7 is an amino acid residue selected from the group consisting of Ile, Met, Gln, Glu, Thr, Leu, Val and Phe.

/8

14. A variant according to claim 13, wherein X^7 is Ile.

15. A variant according to claim 1, wherein X^8 is an amino acid residue selected from the group consisting of Ile, Thr, Leu, Asn, Lys, Ser, Gln, Glu, Arg, Pro and Phe.

/9

16. A variant according to claim 15, wherein X^8 is Ile or Lys.

17. A variant according to claim 1, wherein X^9 is an amino acid residue selected from the group consisting of Arg, Ser, Ala, Gln, Lys and Leu.

20

18. A variant according to claim 17, wherein X^9 is Arg.

19. A variant according to claim 1, wherein X^{10} is an amino acid residue selected from the group consisting of Gln, Pro, Phe, Ile Lys, Trp, Ala, Thr, Leu, Ser, Tyr, His, Asp, Met, Arg and Val. 34

20. A variant according to claim 19, wherein X^{10} is Val or Ile.

21. A variant according to claim 1, wherein X^{11} is an amino acid residue selected from the group consisting of Gly, Met, Gln, Glu, Leu, Arg, Lys, Pro and Asn. 39

22. A variant according to claim 21, wherein X^{11} is Arg or Glu.

23. A variant according to claim 1, wherein X^{12} is Ala or Gly. 40

24. A variant according to claim 1, wherein X^{13} is an amino acid residue selected from the group consisting of Lys, Asn and Asp. 41

25. A variant according to claim 24, wherein X^{11} is Lys or Asn.

26. A variant according to claim 1, wherein X^{14} is an amino acid residue selected from the group consisting of Val, Tyr, Asp, Glu, Thr, Gly, Leu, Ser, Ile, Gln, His, Asn, Pro, Phe, Met, Ala, Arg, Trp and Lys. 46

27. A variant according to claim 26, wherein X^{14} is Lys or Glu.

28. A variant according to claim 1, wherein X^{15} is Lys, Met, Glu or Leu. 52

29. A variant according to claim 1, wherein X^{16} is Lys, Ala, Asn or Glu. 53

30. A variant according to claim 1, wherein X^{17} is Asp. 58

31. A variant according to claim 1, wherein X^1 is Met-His-Ser-Phe

and X^{17} is Asp.

32. A variant according to claim 1 comprising the following amino acid sequence

Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala Arg Ile Ile Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe Val Tyr Gly Gly Cys Arg Ala Lys Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp (SEQ ID No. 2).

33. A DNA construct comprising a DNA sequence encoding a human Kunitz-type protease inhibitor variant according to any of claims 1-32.

34. A recombinant expression vector comprising a DNA construct according to claim 33.

35. A cell containing a DNA construct according to claim 33 or an expression vector according to claim 34.

36. A method of producing a human Kunitz-type protease inhibitor variant according to any of claims 1-32, the method comprising culturing a cell according to claim 35 under conditions conducive to the expression of the protein, and recovering the resulting protein from the culture.

37. A pharmaceutical composition comprising a human Kunitz-type protease inhibitor variant according to any of claims 1-32 and a pharmaceutically acceptable carrier or excipient.

38. A pharmaceutical composition for the inhibition of Cathepsin G, the composition comprising human Kunitz-type protease inhibitor domain I of TFPI or a variant thereof according to any of claims 1-32 and a pharmaceutically acceptable carrier or excipient.

39. A composition according to claim 37 or 38 which further comprises heparin.

40. Use of human Kunitz-type protease inhibitor domain I of TFPI or a variant thereof according to any of claims 1-32 for the preparation of a medicament for the prevention or treatment of diseases or conditions associated with pathological proteolysis.

1/1

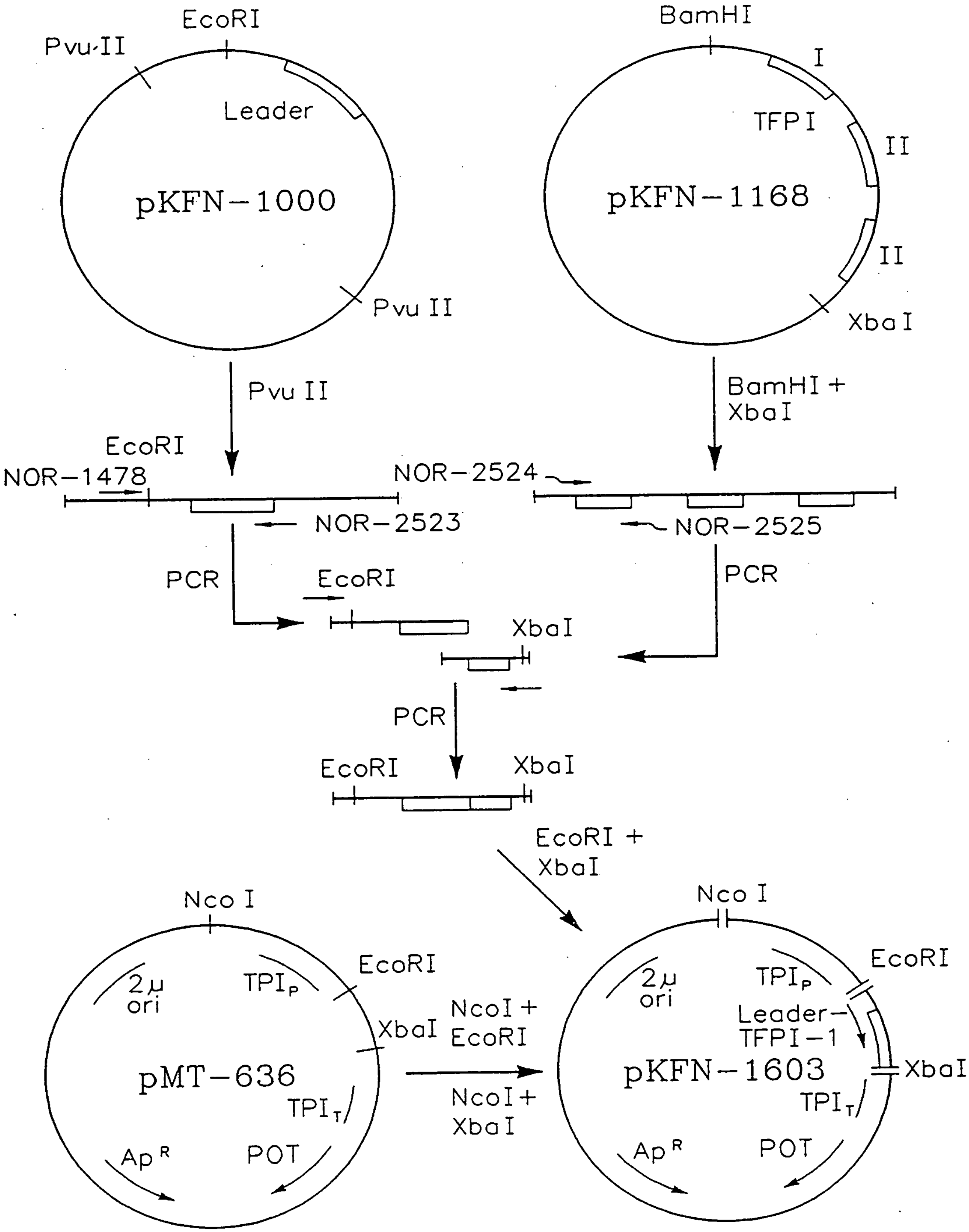
EcoRI
Pvu-II
Leader
pKFN-1000
Pvu II
BamHI
I
TFPI
II
pKFN-1168
II
XbaI
Pvu II
BamHI + XbaI
EcoRI
NOR-1478
NOR-2524
NOR-2523
NOR-2525
PCR
EcoRI
PCR
XbaI
PCR
EcoRI
XbaI
EcoRI + XbaI
Nco I
2μ ori
TPI_P
EcoRI
XbaI
pMT-636
TPI_T
Ap^R
POT
NcoI + EcoRI
NcoI + XbaI
Nco I
2μ ori
TPI_P
EcoRI
Leader-TFPI-1
pKFN-1603
XbaI
TPI_T
Ap^R
POT

Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00005

A. CLASSIFICATION OF SUBJECT MATTER
IPC5: C07K 7/10, C12N 15/15, A61K 37/64 According to International Patent Classification (IPC) or to both national classification and IPC
B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) IPC5: A61K, C07K
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SE,DK,FI,NO classes as above
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CHEMICAL ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE, Volume 338, No --, April 1989, Thomas J. Girard et al, "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor" page 518 - page 520	1,7,8,37,38, 40
A		2-6,9-36
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A,P	US, A, 5106833 (GEORGE J. BROZE, JR. ET AL.), 21 April 1992 (21.04.92)	1-40
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1993	14-04-1993
Name and mailing address of the ISA/	**Authorized officer**
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
26/02/93 PCT/DK 93/00005

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5106833	21/04/92	AU-B- 604138	06/12/90
		AU-A- 1928688	02/02/89
		EP-A- 0300988	25/01/89
		AU-A- 1928788	25/05/89
		EP-A- 0318451	31/05/89